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Form 1/77

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❶ Title of invention

1 Please give the title of the invention: Monoclonal antibodies and their use.

❷ Applicant's details

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2a If you are applying as a corporate body please give:

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Agent's address Coomb House
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5 Are you claiming that this application be treated as having been filed on the date of filing of an earlier application?

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8 Checklist

Please supply duplicates of claim(s), abstract, description and drawing(s).

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Continuation sheets for this Patents Form 1/77	no		
Claim(s)	1	Description	23
Abstract	1	Drawing(s)	10

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Monoclonal antibodies and their use

1. Background

The unique antigenic determinants in and around the antigen-combining site of an Ig molecule which make one antibody distinct from another are defined as idiotopes. The totality of all idiotopes present on the variable portion of a given antibody is referred to as its idiotype (id). The molecular structure of an idiotype has been localized to both the complementarity determining regions and the framework regions of the variable domain and is generally but not always contributed to by both the heavy and the light chains in specific association.

Idiotypes are serologically defined entities since injection of an antibody (often referred to as Ab1) into a syngeneic, allogeneic, or xenogeneic recipient induces the production of anti-idiotypic antibodies (often referred to as Ab2). Niels Jerne (Ann. Immunol. 125C, 373, 1974) postulated a network theory which views the immune system as a collection of Ig molecules and receptors on T lymphocytes, each capable of recognizing an antigenic determinant (epitope) through its combining site (paratope), and each capable of being recognized by other antibodies or cell-surface receptors of the system through the idiotopes that it displays.

When the binding between Ab1 and Ab2 is inhibited by the antigen to which Ab1 is directed, the idiotype is considered to be binding-site-related, since it involves a site on the antibody variable domain that is engaged in antigen recognition. Those idiotopes which conformationally mimic an antigenic epitope are called the internal image of that epitope. Since both an Ab2 and an antigen bind to the relevant Ab1, they may share a similar three-dimensional conformation which represents the internal image.

Monoclonal internal image anti-id antibodies are highly specific reagents for the idiotype of the antibody from which they have been generated. They almost exclusively recognize the binding region of Ab1. This recognition pattern is independent of other determinants of Ab1. In other words, appropriate anti-id Mabs selectively define any molecule which consists of the unique idiotype of Ab1 in its correct three-dimensional shape. Therefore, these anti-id Mabs bind with comparable affinity to $F(ab')_2$-, Fab- and Fv-fragments of Ab1 as well as to its switch variants (switch variants consist of different constant regions, e.g. murine IgG2a, IgG2b, IgG1, but share the identical idiotope).

Mouse/human chimeric Mabs are now often generated by combining the variable domains of the parent murine Mab of choice with human constant regions. Such mouse/human chimeric Mabs may offer advantages for therapeutic uses in man. Internal image anti-id Mabs generated against the parent murine Ab1 also recognize these chimeric antibodies.

The use of monoclonal antibodies for passive immunotherapy is gaining increasing acceptance. One such group of Mabs is BR55-2 and fragments thereof having the same specificity and their variants disclosed in Wistar EP 285 059, M.Blaszcyk-Thurin et al., J. Biol. Chem. 262 (1987) 372-379, or Z. Steplewski et al., Hybridoma 9 (1990) 201-210. These publications also disclose their preparation and their use in the detection and therapy of, basically, adenocarcinomas and similar tumors. The BR55-2 class of antibodies recognizes the Lewis Y6 carbohydrate antigen frequently associated with epithelial cancer.

The present invention comprises the generation, production and characterization of murine monoclonal internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1) and the use of these anti-iditotypic Mabs for the selective and quantitative determination of antibodies or their fragments and derivatives with specificity of BR55-2 as well as a generally applicable method for specific immunoaffinity purification of molecules with specificity of BR55-2.

2. Generation and characterization of murine monoclonal anti-idiotypic antibodies against the idiotype of antibodies BR55-2

In an attempt to minimize undesired anti-isotypic immune responses, the F(ab')$_2$-fragment of BR55-2, murine IgG3, was chosen for immunization. For the successful generation of murine anti-id Mabs against the idiotype of the murine Mab BR55-2, it is important to maximize the immunogenicity in order to raise an appropriate immune response in the syngeneic host. Therefore the F(ab')$_2$-fragment which is devoid of the Fc-part (cleavage and purification described in W 092/03165) was coupled to Keyhole Limpet Hemocyanin (KLH) as immunogenic carrier using the heterobifunctional linker N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP; Pharmacia) according to described methods (J. Carlsson et al., Biochem. J. 173, 723, 1978).

Balb/c mice were immunized with this BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate using Freund's complete adjuvant based on a typical protocol for the generation of murine Mabs. Following repeated immunizations the murine spleen cells were fused with the murine myeloma cell line SP2/0 (for experimental details see example 1).

For an appropriate selection of the cultured hybridoma cells a sequence of tests of their supernatants was performed. This selection was based on the following criteria:

a) Secretion rate of hybridomas by determination of the concentration of murine IgG in the supernatants (for experimental details see example 2). Cells producing high amounts of murine IgG were subcloned to single cell cultures.

b) Binding of selected supernatants to the $F(ab')_2$-fragment of BR55-2/murine IgG3 (for experimental details see example 3).

c) Inhibition of binding of BR55-2/murine IgG2a to Lewis Y antigen positive SKBR5 human breast cancer cells by selected supernatants (for experimental details see example 4).

The latter test is designed to be indicative for internal image properties of Ab2's. The murine IgG2a switch variant of BR55-2 was used for binding in order to minimize detection of Ab2's recognizing remaining constant regions of the $F(ab')_2$-fragment of BR55-2/murine IgG3 used for immunization. This test was performed in a quantitative manner based on the IgG concentration determined in test a) (example 2). Furthermore an excess of unspecific mouse IgG was added to this inhibition experiment in order to avoid any detection of Ab2's not specific for the idiotype of BR55-2.

Hybridomas were chosen which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2/murine IgG2a to the SKBR5 cell line).

Using the test procedures mentioned above six different hybridomas were finally selected and expanded (E4, C11, B3, B9, G6, G9). All six hybridomas produce murine IgG1 as detected by subtype ELISA using rabbit-anti-mouse IgG1/peroxidase (such as the reagent of Zymed).

All six hybridomas were cultured in roller flasks (37° C, 5% CO_2 in medium G; change of medium every 3 to 4 days) and the supernatants were collected for subsequent purification.

Each supernatant containing the respective anti-Id BR55-2 Mab was purified using immunoaffinity chromatography. In general, affinity chromatography is based on the interaction between an immobilized ligand and the substance of interest. In the case of anti-idiotypic BR55-2 Mabs, the highly specific ligand for the affinity column is Mab BR55-2/murine IgG2a which binds the anti-idiotypic Mabs of choice (for experimental details see example 5).

The degree of purity of the isolated anti-id BR55-2 Mabs (E4, C11, B3, B9, G6, G9) was tested by analytical FPLC ion-exchange-chromatography, size-exclusion-chromatography, SDS-PAGE and isoelectric focussing. Purity of all six anti-id BR55-2 Mabs was >95% (for experimental details see example 6; SDS-PAGE and isoelectric focussing are shown in figures 1 and 2).

The purified anti-id Mabs were quantitatively characterized by determination of their capacity to inhibit binding of BR55-2/murine IgG3 to the Lewis Y antigen positive SKBR5 cell line. All anti-id Mabs inhibit the binding of Ab1 to its antigen based on a 1:1 stoechiometry (for experimental details see example 7; representative results are shown in figure 3).

The internal image anti-idiotypic Mabs raised against the binding region of BR55-2 described above are powerful tools for the quantitative determination of monoclonal antibodies, their

derivatives or fragments with binding specificity of BR55-2. For example they can be used for the selective determination of the concentration of all murine subtypes of BR55-2 in human sera. Because of the recognition of the three-dimensional shape of the binding region of BR55-2 these anti-id Mabs only detect immunoreactive BR55-2 structures. An ELISA-system for the quantitative determination of BR55-2/murine IgG3 or BR55-2/murine IgG2a is described in example 8 and a typical standard curve in human serum is shown in Figure 4.

A similar ELISA-system based on these anti-id Mabs can be applied for the highly selective quantitative determination of mouse/human chimeras of BR55-2 in human serum. These chimeras consist of the variable domains of BR55-2 and of human IgG1 or human IgG3 constant regions. Their preparation and properties are published (7th Intern. Conf. on Monoclonal Antibody Immunoconjugates for Cancer, San Diego, 5.-7.3.1992). Because of the huge excess of normal human Ig in human serum (>10 mg/ml), such mouse/human chimeras cannot be detected in human serum using conventional anti-human-Fc reagents which bind to any human Ig present. The selective ELISA-system is described in examples 9 and 10, typical standard curves in human serum are shown in Figures 5 and 6.

Two fully humanized variants of BR55-2 have been constructed by grafting of the complementarity determining regions (CDRs) of the murine parent Mab to human framework and constant regions. By means of computer modelling some point mutations in the human framework chosen lead to humanized variants without substantial loss in binding affinity in comparison to the parent murine Mab (for experimental details see example 15). Thus, only the small portion of the original murine amino acid sequences determining the binding characteristics remains unchanged in these humanized variants. Remarkably the anti-id BR55-2 Mabs also bind strongly to the idiotype of both humanized IgG1 variants. This highlights the binding selectivity of the anti-id Mabs to the three dimensional shape of the hypervariable region of all variants of BR55-2 and their internal image properties.

Based on this unique and selective recognition pattern the anti-id BR55-2 Mabs can also be used for the highly selective quantitative determination of fully humanized antibodies with specificity of BR55-2 in human serum despite of the huge excess of human immunoglobulin. This ELISA-system is described in example 11, a typical standard curve in human serum is shown in Figure 7.

The recognition of the binding region of all variants of BR55-2 by anti-id BR55-2 Mabs is also demonstrated by the competition of binding of BR55-2/murine IgG3 to anti-id BR55-2 #E4 by chimeric human IgG1 and chimeric human IgG3. This competition ELISA is described in example 12 and the results are shown in Figure 8.

Binding of BR55-2/murine IgG3 to antigen positive tumor cell lines is a prerequisite for complement mediated destruction. Thus, inhibition of tumor cytotoxicity by anti-id BR55-2 Mabs reflect their ability to block the binding of Ab1 to its antigen on the cell surface. The inhibition by anti-id BR55-2 #E of complement dependent cytotoxicity to SKBR5 human breast cancer cells mediated by BR55-2/murine IgG3 is shown in Figure 9, for experimental details see example 13.

Due to the highly specific recognition of all structures with intact binding region of BR55-2 the anti-id BR55-2 Mabs can be used for a single step immunopurification procedure of variants of BR55-2. Anti-id BR55-2 #E4 has been coupled to CH-Sepharose 4B. This immunoaffinity material for example can be used for a straight forward purification of BR55-2/chimeric human IgG1 cultured in vitro. The chimeric Mab is obtained in a yield of 75% with a purity of >95%. For experimental details see example 14, SDS-PAGE is shown in Figure 10. This one step immunopurification method is equally suitable for fully humanized variants of BR55-2 obtained by grafting of CDRs (generation of fully humanized variant is described in example 15).

The following examples illustrate the invention. The abbreviations have the following meanings:

BSA:	bovine serum albumin
CDC:	complement dependent cytotoxicity
DMEM:	Dulbecco modified Eagle Medium
ELISA:	enzyme-linked immunosorbent assay
FCS:	fetal calf serum
Mab:	monolonal antibody
PBS:	phosphate-buffered saline
RPMI:	Rosewell Park Memorial Institute
SDS:	sodium dodecyl sulfate
KLH:	keyhole limpet hemocyanin
SPDP:	N-succinimidyl-3-(2-pyridyl-dithio-propionate)
PAGE:	polyacrylamide gel electrophoresis
IEF:	isoelectric focussing
PEG:	polyethyleneglycol
EDTA:	ethylene diamine tetraacetic acid
FPLC:	fast protein liquid chromatography
PCR:	polymerase chain reaction
DTT:	dithiothreitol
dNTP:	desoxynucleotide-5'-triphosphate

TdT: terminal deoxynucleotidyl transferase

dGTP: desoxyguanosine-5'-triphosphate

CDR: complementary determining region

The materials referred to in the examples are as follows:

Microtiterplates: Immunoplates II (Nunc)
tissue culture treated flat bottom plates

Cell lines: SKBR5: human breast cancer cell line
SP2/0: mouse myeloma cell line

Medium A: RPMI 1640 + 2 g/l $NaHCO_3$
100 U/ml penicillin G
100 µg/ml streptomycin sulfate
4 mM glutamine
10 % FCS (heat-inactivated, γ-globulin-free)

Medium B: RPMI 1640 + 2 g/l $NaHCO_3$
100 U/ml penicillin G
100 µg/ml streptomycin sulfate
4 mM glutamine
5 % FCS (heat-inactivated)

Medium C: DMEM
10 % NCTC-135 (synthetic medium, Gibco)
1 % MEM non essential amino acids (Gibco)
0.5 % sodium pyruvate
0.5 % oxalacetic acid (Sigma)
20 % FCS (heat-inactivated)
4 mM glutamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

Medium D: Medium C + 1.36 mg/l hypoxanthine
0.39 mg/l thymidine

Medium E: Medium D + 0.4 mg/l aminopterin

Medium F: Medium C + mouse thymocytes (thymocytes of one Balb/c mouse resuspended in 25 ml medium C)

Medium G: DMEM
10 % FCS (heat-inactivated)

4 mM gluatamine
100 U/ml penicillin G
100 µg/ml streptomycin sulfate

PEG: polyethyleneglycol (MW = 3400)
1 g is dissolved in 1 ml DMEM

PBS deficient: 138.0 mM NaCl
1.5 mM KOH
2.7 mM KCl
6.5 mM Na_2HPO_4
pH 7.2

Coating buffer: 15 mM Na_2CO_3
35 mM $NaHCO_3$
3 mM NaN_3
pH 9.6

Staining buffer: 24.3 mM citric acid
51.4 mM Na_2HPO_4
pH 5.0

Washing buffer: 2% NaCl
0.2 % Triton X-100
in PBS deficient

Substrate solution: 40 mg o-phenylenediaminedihydrochloride
100 ml staining buffer
20 µl H_2O_2 30%

Binding buffer: 0.1 M Tris/HCl
0.2 M NaCl
pH 7.5

Elution buffer: 0.15 M glycine/HCl
0.2 M NaCl
pH 2.8

Coupling buffer: 0.1 M $NaHCO_3$
0.5 M NaCl
pH 8.0

$Na_2{}^{51}CrO_4$: 1 mCi/ml

In the following examples which illustrate the invention but in no way limit its scope references to temperature are in degrees celsius.

Example 1: Generation of anti-Id BR55-2 #E4

1.1 Immunization of mice

Balb/c mice are immunized with each 100 µg of $F(ab')_2$-fragment of BR55-2/murine IgG3, coupled to KLH via SPDP as described (J. Carlsson et al., Biochem. J. 173, 723, 1978) by intraperitoneal injection in the following scheme:

day 0: 100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's complete adjuvant

day 7 and 28: 100 µg of conjugate (1 mg/ml in PBS def.) + 100 µl of Freund's incomplete adjuvant

On days 8, 9, 10 and 11 after primary immunization i.v. a total of 4 boost injections (each 100 µg of conjugate in 100 µl of PBS def.) are given.

On day 12 the spleens are taken out aseptically, suspended in PBS def. and washed thrice in PBS def.

1.2 Hybridization

These spleen cells are added to a suspension of SP2/0 cells in a ratio 1:1 and centrifuged at 900 g for 5 minutes. 1 ml of PEG-solution (37°) is added dropwise to the cell pellet within 1 minute and diluted with 1 ml of PBS def. (37°) within the next minute. 10 ml of medium C are added under gently rotation and the suspension is diluted to 50 ml with PBS def. The suspension is centrifuged at 800 g for 5 minutes, the pellet resuspended in medium D and the cells are transferred into the wells of a microtiterplate (Nunc 96) at a concentration of 2.5×10^5 cells/well. After overnight incubation at 37°/5% CO_2 100 µl/well of medium E are added. After 72 hours and then every four days the medium is replaced by medium D.

Example 2: Quantitative determination of mouse IgG in hybridoma supernatants

100 µl aliquots of rabbit-anti-mouse IgG (such as the reagent of Nordic; 1:1000 in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl aliquots of the hybridoma supernatants obtained after 2 weeks culture are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2% FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4N H_2SO_4. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 3: Specific binding of hybridoma supernatant-IgG to BR55-2 F(ab')$_2$-fragment (ELISA)

Hybridomas producing sufficient mouse IgG (i.e. more as 10-fold optical density than the medium-blank) are subcloned to single cell culture in medium F and cultured in medium G for additional 2 weeks. The supernatants are tested as follows:

100 µl aliquots of F(ab')$_2$-fragment of BR55-2 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates, and incubated at 37° for 60 minutes.

The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. 100 µl of hybridoma supernatants are added and the plates are incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG-Fc/peroxidase such as the reagent of Dianova; 1:1000 in PBS/2 % FCS) are added.

After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer.

100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Photometric extinction is measured at 492 nm (reference measurement 620 nm).

Example 4: Inhibition of binding of BR55-2/murine IgG2a to SKBR5 human breast cancer cells by hybridoma supernatant-IgG (cell ELISA)

All hybridoma supernatants which are positive in the above described assay are tested as follows:

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD; 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml).

After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1% in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05% Tween 20. Hybridoma supernatants adjusted to 1 µg/ml mouse IgG are preincubated with 10-fold excess of unspecific mouse IgG for 30 minutes at 37°. Then these samples are preincubated with 0.5 µg/ml of BR55-2/murine IgG2a for 30 minutes at 37°. 100 µl of this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG2a/peroxidase such as the reagent of Zymed; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes the colour development is stopped by addition of 50 µl aliquots of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 5: Immunoaffinity purification of anti-id BR55-2 Mabs

5.1 Preparation of BR55-2/murine IgG2a Sepharose

10 g of freeze dried activated CH-Sepharose 4B is suspended in 1mM HCl, transferred to a sinter glass filter and washed with 2 l of 1 mM HCl for 15 minutes. The ligand (120 mg of BR55-2/murine IgG2a) dissolved in 50 ml of coupling buffer is mixed with the washed gel in a stoppered vessel and rotated end over end for one hour at room temperature. The gel is washed with coupling buffer and incubated for one hour with 50 ml of 1M ethanolamine for blocking of any remaining active groups. The affinity sorbent is then washed with three cycles of alternating pH. Each cycle consists of a wash at pH 4 (0.1 M acetate, 0.5 M NaCl) followed by a wash at pH 8 (0.1 M Tris, 0.5 M NaCl).

5.2 Isolation of the anti-id BR55-2 Mabs

The chromatography is performed at 4°. The column (BIO REX MP column, diameter 1.5 cm) is filled with Mab BR55-2/murine IgG2a Sepharose (volume 35 ml). The gel is washed with binding-buffer and elution buffer. After equilibration with binding buffer conditioned medium containing anti-id BR55-2 is loaded onto the column at a flow rate of 15 ml/min. After elution of the breakthrough fraction, the bound anti-id BR55-2 is desorbed with elution buffer and neutralized immediately after desorption with 1 M Tris/HCl buffer, pH 7.5.

5.3 Concentration of the anti-id BR55-2 Mabs

Concentration of the eluted antibody solution (0.12 mg/ml) is performed in a stirred Amicon ultrafiltration cell using a PM 10 Diaflo membrane. The solute rejection for IgG is more than 98%, the final concentration of IgG amounts to 3.7 mg/ml.

Example 6: Characterization of purified anti-id BR55-2 Mabs

6.1 Ion-exchange-chromatography on Mono-Q

Column:	Mono-Q HR5/5 (Pharmacia)
Buffer A:	20 mM tri-ethanolamine, pH 7.7
Buffer B:	20 mM tri-ethanolamine, 1 M NaCl, pH 7.7
Flow rate:	1 ml/min
Detection:	UV 280 nm
Gradient:	linear 2%/min
Results:	> 95% purity found for all anti-id BR55-2 Mabs

6.2 High performance size-exclusion-chromatography

Column:	Zorbax GF250, 9.4 x 250 mm
Buffer :	Sodium phosphate 0.1 M, 0.2 M NaCl, pH 7.0
Flow rate:	1 ml/min
Detection:	UV 280 nm
Results:	> 95% purity found for all anti-id BR55-2 Mabs

6.3 SDS-PAGE

Experiments are performed both under reducing and non-reducing conditions according to the method of Laemmli using 10 % acrylamide gels (results are shown in Figure 1).

6.4 Isoelectric focussing

Analysis is performed with the Phast-system (Pharmacia) using a pH-gradient 3-9 (Phast gel IEF 3-9) and silver staining for detection of the protein bands (results are shown in figure 2).

Example 7: Binding of BR55-2/murine IgG3 to SKBR5 cell line (cell-ELISA) - inhibition by anti-id BR55-2 #E4

Microtiter plates are pretreated with poly-L-lysine hydrobromide (20-30 kD: 20 µg/ml in PBS def.; 100 µl/well; 30 minutes, room temperature), washed twice with PBS def. (200 µl/well) and then incubated overnight at 4° with 50 µl/well of a suspension of SKBR5 cells in medium B ($4x10^6$ cells/ml). After removal of the supernatant the cells are fixed with 50 µl of glutardialdehyde/well (0.1 % in physiological saline) for 5 minutes at room temperature, the supernatants are removed, the cells resuspended in 200 µl/well of PBS def./1% BSA/0.1% NaN_3 and left for 1 hour at room temperature.

Supernatants are removed and the plates are washed twice with 200 µl/well of PBS containing 0.05 % Tween 20. Anti-id BR55-2 #E4 is diluted in PBS def. containing 2% FCS (10 to 0.5 µg/ml). To each of these dilutions 1 µg/ml of BR55-2/murine IgG3 is added. 100 µl of

this mixture are added to the cells and the plates are incubated for 1 hour at 37°. Unbound antibody is washed out twice with 100 µl/well of ice-cold PBS containing 0.05 % Tween 20. 100 µl aliquots of peroxidase-conjugated antibody (rabbit anti-mouse IgG3/peroxidase such as the reagent of Zymed; 1:1000 in PBS def./2% FCS) are added. After incubation for 45 minutes at 37° the wells are washed thrice with the PBS/Tween 20 solution mentioned above and then 100 µl of the substrate solution are added to each well. After 5 minutes colour development is stopped by addition of 50 µl of 4 N H_2SO_4/well. Binding of the antibody to the cells is determined by measuring extinction at 492 nm (reference measurement 620 nm).

Example 8: ELISA for the determination of BR55-2/murine IgG3 or BR55-2/murine IgG2a in human serum using anti-id BR55-2 #E4

100 µl aliquots of anti-id BR55-2 #E4 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates and incubated at 37° for 60 minutes. The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. Human sera containing BR55-2/murine IgG3 or BR55-2/murine IgG2a are tested in appropriate dilutions in PBS def./2% FCS. 100 µl aliquots of these samples are added to the wells of the microtiter plates and incubated for 60 minutes at 37°. As standard BR55-2/murine IgG3 or BR55-2/murine IgG2a is prediluted in normal human serum to 10 µg/ml. Appropriate dilutions in PBS def./2% FCS are treated as above. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (rabbit-anti-mouse IgG3/peroxidase or rabbit-anti-mouse IgG2a/peroxidase such as the reagent of Zymed, 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm). The OD values of the serum samples are read on the standard curve and expressed in µg/ml.

Example 9: ELISA for the determination of BR55-2/chimeric human IgG1 in human serum using anti-id BR55-2 #E4

100 µl aliquots of anti-id BR55-2 #E4 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates and incubated at 37° for 60 minutes. The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. Human sera containing BR55-2/chimeric human IgG1 are tested in appropriate dilutions in PBS def./2% FCS. 100 µl aliquots of these samples are added to the wells of the microtiter plates and incubated for 60 minutes at 37°. As standard BR55-2/chimeric human IgG1 is prediluted in normal human serum to 10 µg/ml.

Appropriate dilutions in PBS def./2% FCS are treated as above. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human IgG/peroxidase such as the reagent of Chemicon & Co., 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm). The OD values of the serum samples are read on the standard curve and expressed in µg/ml.

Example 10: ELISA for the determination of BR55-2/chimeric human IgG3 in human serum using anti-id BR55-2 #E4

100 µl aliquots of anti-id BR55-2 #E4 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates and incubated at 37° for 60 minutes. The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. Human sera containing BR55-2/chimeric human IgG3 are tested in appropriate dilutions in PBS def./2% FCS. 100 µl aliquots of these samples are added to the wells of the microtiter plates and incubated for 60 minutes at 37°. As standard BR55-2/chimeric human IgG3 is prediluted in normal human serum to 10 µg/ml. Appropriate dilutions in PBS def./2% FCS are treated as above. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human IgG/peroxidase such as the reagents of Chemicon & Co., 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm). The OD values of the serum samples are read on the standard curve and expressed in µg/ml.

Example 11: ELISA for the determination of BR55-2/humanized IgG1 in human serum using anti-id BR55-2 #E4

100 µl aliquots of anti-id BR55-2 #E4 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates and incubated at 37° for 60 minutes. The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. Human sera containing BR55-2/humanized IgG1 are tested in appropriate dilutions in PBS def./2% FCS. 100 µl aliquots of these samples are added to the wells of the microtiter plates and incubated for 60 minutes at 37°. As standard BR55-2/humanized IgG1 is prediluted in normal human serum to 10 µg/ml. Appropriate dilutions in PBS def./2% FCS are treated as above. Unbound antibody is washed out as described

above and 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human IgG/peroxidase such as the reagent of Chemicon & Co., 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm). The OD values of the serum samples are read on the standard curve and expressed in µg/ml.

Example 12: Competition of binding of BR55-2/murine IgG3 to anti-id BR55-2 #E4 by chimeric human IgG1 or chimeric human IgG3

100 µl aliquots of anti-id BR55-2 #E4 (10 µg/ml; dilution in coating buffer) are added to the wells of microtiter plates and incubated at 37° for 60 minutes. The plates are washed 6 times with washing buffer, 200 µl of PBS def./5% FCS are added and incubated for 30 minutes at 37°. The plates are washed as described above. Chimeric human IgG1 or chimeric human IgG3 is diluted in PBS def./2% FCS (0.5 µg/ml to 3 ng/ml). To each dilution, 0.05 µg/ml BR55-2/murine IgG3 are added. 100 µl aliquots of these mixture are added to the wells of the microtiter plates and incubated for 60 minutes at 37°. Unbound antibody is washed out as described above and 100 µl aliquots of peroxidase-conjugated antibody (goat-anti-human IgG/peroxidase such as the reagent of Chemicon Co., 1:1000 in PBS/2% FCS) are added. After incubation for 30 minutes at 37° the plates are washed 4 times with washing buffer and twice with staining buffer. 100 µl aliquots of substrate solution are added and colour development is stopped after 5 minutes with 50 µl aliquots of 4 N H_2SO_4/well. Optical density (OD) is measured at 492 nm (reference measurement 620 nm).

Example 13: Complement dependent cytotoxicity (CDC) to SKBR5 cell line mediated by BR55-2/murine IgG3 - inhibition by anti-id BR55-2 #E4

On the day preceding the assay the SKBR5 cells are transferred into fresh medium A and kept at 37°/5% CO_2 in a cell culture flask.

^{51}Cr labelling of the target cells:

The cells are collected from the culture flask and incubated at a concentration of $5x10^6$ cells in 800 µl of medium A at 37°/5% CO_2 for 1 hour with 100 µCi $Na_2{}^{51}CrO_4$. The cells are then washed with medium A to remove the excess ^{51}Cr, resuspended in fresh medium A, and their concentration is adjusted to $2.5x10^5$ cells/ml.

CDC:

100 µl aliquots of this suspension of target cells are pipetted into the wells of microtiter plates. 50 µl aliquots of anti-id BR55-2 #E4 diluted to the desired concentrations in PBS def. are

added. Then 100 µl aliquots of a human serum containing 2 µg/ml of BR55-2/IgG3 are added per well and the cells are incubated overnight at 37°/5% CO_2. The supernatants are harvested with a Skatron-Harvesting-Press and counted in a γ-counter. This yields the value for the experimental release.

For determination of total ^{51}Cr release the cells are treated as above replacing the human serum by a solution of 2% SDS, 50 mM Na_2CO_3 and 10 mM EDTA and the anti-id BR55-2 #E4 solution by 50 µl PBS def. The value for spontaneous ^{51}Cr release is obtained by replacing the human serum by medium A and the anti-id BR55-2 #E4 solution by 50 µl PBS def.

After counting the result is calculated as follows:

$$\% \text{ lysis} = \frac{(\text{experimental release} - \text{spontaneous release}) \times 100}{\text{total release} - \text{spontaneous release}}$$

Example 14: Immunoaffinity purification of BR55-2/chimeric human IgG1 using anti-id BR55-2 #E4 -Sepharose

14.1. Preparation of anti-id BR55-2 #E4 -Sepharose

9 g of freeze dried activated CH-Sepharose 4B is suspended in 1mM of HCl, transferred to a sinter glass filter and washed with 2 l of 1 mM HCl for 15 minutes. The ligand (95 mg of anti-id BR55-2 #E4) dissolved in 50 ml of coupling buffer is mixed with the washed gel in a stoppered vessel and rotated end over end for one hour at room temperature. The gel is washed with coupling buffer and incubated for one hour with 50 ml of 1 M ethanolamine for blocking of any remaining active groups. The affinity sorbent is then washed with three cycles of alternating pH. Each cycle consists of a wash at pH 4 (0.1 M acetate, 0.5 M NaCl) followed by a wash at pH 8 (0.1 M Tris, 0.5 M NaCl).

14.2. Isolation of the BR55-2/chimeric human IgG1

The chromatography is performed at 4°. The column (BIO REX MP column, diameter 1.5 cm) is filled with anti-id BR55-2 #E4 -Sepharose (volume 35 ml). The gel is washed with binding buffer and elution buffer. Starting material are 100 l of BR55-2/chimeric human IgG1 in conditioned medium. After concentration 5:1 using an Amicon DC2 concentration system equipped with a hollow fiber cartridge P10, the concentrate was loaded onto the column. After elution of the breakthrough fraction, the bound BR55-2/chimeric human IgG1 is desorbed with elution buffer and neutralized immediately after desorption with 1 M Tris/HCl buffer, pH 7.5.

14.3. Concentration of the BR55-2/chimeric human IgG1

Concentration of the eluted antibody solution is performed in a stirred Amicon ultrafiltration cell using a PM 10 Diaflo membrane. The solute rejection for IgG is more than 98%, the final concentration of IgG amounts to 3.36 mg/ml

14.4. Characterization of purified BR55-2/chimeric human IgG1

14.4.1. Ion-exchange-chromatography on Mono-Q

Column:	Mono-Q HR5/5 (Pharmacia)
Buffer A:	20 mM triethanolamine, pH 7.7
Buffer B:	20 mM triethanolamine, 1 M NaCl, pH 7.7
Flow rate:	1 ml/min
Detection:	UV 280 nm
Gradient:	linear 2%/min
Result:	purity found >95%

14.4.2. High performance size-exclusion-chromatography

Column:	Zorbax GF250, 9.4 x 250 mm
Buffer:	Sodium phosphate 0.1 M, 0.2 M NaCl, pH 7.0
Flow rate:	1 ml/min
Detection:	UV 280 nm
Result:	purity found >95%

14.4.3. SDS-PAGE

Experiments are performed under reducing conditions according to the method of Laemmli using 10% acrylamide gels (results are shown in Figure 10).

Example 15: Process for production of fully humanized monoclonal antibodies BR55-2:

15.1. Cloning and sequencing of the heavy chain and light chain variable domain cDNA for the BR55-2/murine IgG3

The variable domain cDNA for the heavy chain and light chain of BR55-2/murine IgG3, was cloned by the anchored PCR method. First, a total RNA preparation was prepared using the hot phenol method. Briefly, $1x10^7$ BR55-2/murine IgG3, hybridoma cells were resuspended in 1.2 ml of RNA extraction buffer (50 mM sodium acetate, pH 5.2, 1% SDS), vortexed and incubated at room temperature for 2 min. The cell lysates were then incubated with 0.6 ml of phenol, pH 5.2, at 65°C for 15 min, followed by another 15 min incubation on ice. The extract was spun in a microfuge; the aqueous phase was recovered and ethanol precipitated twice. The RNA pellet was resuspended in water and quantitated at OD_{260}. cDNA was synthesized from the total RNA using reverse transcriptase (5 µg total RNA, 40 ng dT_{12-18} (Pharmacia), 200 units of M-MLV reverse transcriptase (BRL), 40 units of RNAsin (Pomega), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$ and 0.5 mM each dNTP in a 20 µl reaction volume). The G-tailing was achieved with terminal deoxynucleotidyl transferase (TdT)

(cDNA, 15 units TdT (BRL), 0.1 M potassium cacodylate, pH 7.2, 2 mM $CoCl_2$, 0.2 mM DTT and 1 mM dGTP in a 20 µl reaction volume). Under the conditions described, tails generally contained about 20 bases. One half of the G-tailed product was then amplified to generate the V_L gene and the other half amplified to provide the V_H gene using Taq polymerase. The V_L gene is amplified with the primer mc45 that anneals to the G-tail and a primer mc46 that anneals to the constant region of the kappa light chain. The V_H gene was amplified with primers mc45 and mc47 that anneals to the constant region of gamma chains. EcoRI and HindIII sites are included in the upstream and downstream primers for convenient subcloning into pUC18 vector. An alternate set of restriction sites (XbaI and SacI) are also included in the primers for the rare event that EcoRI and HindIII sites are present in the variable region genes. The PCR reactions were performed in a programmable heating block using 30 rounds of temperature cycling (92° for 1 min, 50° for 2 min and 72° for 3 min). The reaction included the G-tailed product, 1 µg of each primer and 2.5 units of Taq polymerase (Perkin Elmer Cetus) in a final volume of 100 µl, with the reaction buffer recommended by the manufacturer. The PCR product bands were excised from a low-melting agarose gel, digested with restriction enzymes and cloned into pUC18 vector for sequence determination.

15.2. Construction of expression vectors

Separate expression vectors were used to express the humanized BR55-2 IgG1 antibody light and heavy chains: pVk for the light chain, pVg1 for the gamma 1 heavy chain. First pVk and then pVg1 are described in detail below; nucleotide position numbers start with 1 at the EcoRI site and refer to the complete plasmids.

Plasmid pVk. Proceeding clockwise, pVk first contains the human cytomegalovirus (CMV), major immediate early (IE) enhancer and promoter. The function of the promoter is to initiate transcription of the light chain gene cloned at the XbaI site, and the function of the enhancer is to strongly increase the level of transcription. Thus the part of human CMV used is regulatory; no proteins are encoded. The CMV region is preceded by a short oligonucleotide linker used to connect it to the EcoRI site of the preceding pBR322 fragment.

The CMV region is followed by another linker containing an XbaI site. The variable region of an antibody light chain gene such as humanized BR55-2 may be cloned into the XbaI site. The XbaI site is followed by part of a genomic clone of the human kappa light chain constant region (C_L), including the coding sequence, polyadenylation (poly A) signal, and part of the preceding intron.

The C_L region is followed by a gene encoding xanthine guanine phosphoribosyl transferase (gpt), together with regulatory elements (enhancer, promoter, splice signals, poly A signal)

from Simian Virus 40 (SV40) needed for transcription. The function of this region, which was taken as a unit from the plasmid pSV2-gpt, is to provide a selectable drug-resistance marker after transfection of pVk into mammalian cells. Moving counter-clockwise within this one unit, first there is a SV40 segment containing the SV40 enhancer and early promoter, to ensure strong transcription initiation. This segment is followed by the coding sequence of the E.coli gpt gene. The gpt gene is followed by a SV40 segment containing the small t antigen intron, believed to increase mRNA levels, and then another SV40 segment containing a poly A signal for ending the mRNA transcript. The direction of transcription of the gpt gene is opposite to that of the kappa light chain gene. Finally, pVk contains a large part of the widely used E.coli vector plasmid pBR322, comprising the origin of replication and ampicillin resistance gene (amp), respectively used for growth and selection in E.coli. These procaryotic elements are expected to be non-functional after pVk is transfected into mammalian cells.

Plasmid pVg1. This plasmid is similar to pVk but contains a heavy chain instead of light chain constant region and a different selectable marker. Specifically, proceeding clockwise, pVg1 contains the same CMV enhancer and promoter for strong transcription initiation as pVk, inserted with the same EcoRI and XbaI linkers. The variable region of an antibody heavy chain such as humanized BR55-2 can be inserted at the XbaI site. That site is followed by part of a genomic clone containing the human gamma 1 heavy chain constant region (C_H) including the C_H1, hinge (H), C_H2 and C_H3 exons with the intervening introns, part of the intron preceding C_H1, and a poly A site following C_H3. The C_H region is followed by a gene encoding a mutant gene for dihydrofolate reductase (dhfr), together with regulatory elements (enhancer, promoter, splice signals, poly A signal) from SV40 needed for transcription. This unit is identical to the gpt unit in pVk, except that dhfr replaces gpt. The mutant dhfr gene confers resistance to methotrexate, so it can be used as a selectable marker after transfection into mammalian cells. The mutant dhfr was cloned from a wide-type gene with a single amino acid substitution at position 22 (Leu to Arg) and can be employed as a dominant selectable marker in cultured cells expressing normal levels of wide-type dihydrofolate reductase. This marker also allows to select higher antibody producers by subjecting cells to increased level of methotrexate. Finally, pVg1 contains the same part of the plasmid pBR322 as pVk, containing the origin or replication and amp gene for use in E.coli.

15.3. Computer modeling of humanized variable region domain

In order to retain high binding affinity in the humanized antibody, the general procedures of Queen et al. were followed. The first step in the designing of humanized antibody is to perform a sequence homology search to select the best framework. Comparison of variable regions

of BR55-2, murine IgG3, with a few selected human antibodies is shown below (sequence homology including CDRs given in percentage):

Ab	VL	VH
Eu	54%	43%
Sie	57%	41%
Ou	48%	41%
Lay	56%	64%
Pom	56%	64%
Tew	77%	-

Pom was selected to provide the framework for the humanized heavy chain and Tew for the humanized light chain variable region.

Next, the computer program ABMOD and ENCAD were used to construct a molecular model of the BR55-2, murine IgG3, variable domain. Inspection of the refined model of murine BR55-2 revealed several amino acid residues in the framework that have significant contacts with the CDR residues (category 4 below). To design the humanized light and heavy chain BR55-2 variable regions, at each position the amino acid was chosen to be the same as in the Tew or Pom sequence, respectively, unless that position fell in one or more of the four categories:

(1) The position fell within a CDR.

(2) The Pom or Tew amino acid was unusual for human antibodies at that position, whereas the BR55-2, murine IgG3, amino acid was typical for human antibodies at that position.

(3) The position was immediately adjacent to a CDR.

(4) The model described above suggested that the amino acid may be physically close to the antigen binding region (CDRs).

Category	Light Chain	Heavy Chain
1	24-39, 55-61, 94-102	31-35, 50-66, 99-108
2	108	82, 87
3		109
4	54	73, 74, 109

The humanized light chain and heavy chain sequences are labeled L-hu-BR55-2 and H-hu-BR55-2/1, respectively. Binding affinity measurements showed that the binding affinity of the humanized antibody is roughly four fold lower than that of the mouse/human chimeric IgG1 antibody.

Since the overall electrostatics of a protein can affect the binding of a substrate, this effect was investigated in an effort to increase the binding affinity of the humanized antibody. The amino acid sequences of the humanized heavy chain and the murine heavy chain were compared to identify framework residue differences that result in a charge change. Several humanized heavy chain variants with single or double amino acid substitutions from the murine sequence were constructed. One variant with substitutions at position 42 (Gly to Glu) and position 44 (Gly to Arg) increases the binding affinity by two fold. One variant with a substitution at position 95 (Tyr to His), which is involved in the interfacing with the light chain, also increases binding affinity by two fold. A variant chain, incorporating these three substitutions, the sequence of which is labeled H-hu-BR55-2/2, was constructed and shown to bind to the antigen with affinity within two fold of the mouse/human chimeric IgG1 antibody.

It was also found that substituting residue 75 in the heavy chain with the murine residue enhances antibody secretion. The humanized heavy chain sequence, which incorporates this additional change, is labeled H-hu-BR55-2/3. The heavy chains described above were then cotransfected each with the L-hu-BR55-2 light chain to produce the respective humanized antibodies.

The variants with the heavy chain sequence H-hu-BR55-2/2 and H-hu-BR55-2/3 were named humanized BR55-2/2 and humanized BR55-2/3 respectively.

15.4. Construction of variable domain segments.

Humanized BR55-2 light chain. The actual expressed humanized BR55-2 light chain gene consists of two adjacent parts: a human genomic kappa constant region built into the vector pVk (see above), and the humanized light chain variable region (V_L) constructed by total gene synthesis from oligonucleotides.

For the construction of light chain variable region gene, nucleotide sequences were selected that encode the protein sequences of the humanized light chain, including the signal peptide, generally utilizing codons found in the mouse sequence. Several degenerate codons were changed to create restricition sites or to remove undesirable ones. The nucleotide sequences also included a splice donor signal from the J_K4 in the mouse genomic sequence and an XbaI site at each end. The gene was constructed from four overlapping synthetic oligonucleotides. For the variable domain gene, two pairs of overlapping synthetic oligonucleotides on alternating strands were synthesized that encompassed the entire coding sequences as well as the signal peptide and the splice donor signal. The oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. Each oligo was about 110-140 base long with a 15 base overlap. Double stranded DNA fragments were synthesized with Klenow polymerase, digested with

restriction enzymes, ligated to pUC18 vector and sequenced. The two fragments with the correct sequences were then ligated into the Xbal sites of pVk expression vector.

Thus, tne cloned segment encodes the humanized V_L domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the light chain is secreted. In addition, the segment includes the same 23 base pairs after the J segment that follow the mouse J_K4 segment. The purpose of these nucleotides is to provide a splice donor signal to ensure that the intron between the V_L region and the downstream C_L region is correctly spliced out. The correct orientation and sequence of the complete variable region (V_L) segment in pVk was then verified by sequencing again. All manipulations were done by standard methods.

Thus, the complete humanized BR55-2 light chain gene consists of the segment between a Xbal and a BamHI site. It contains a variable region exon (including leader and J segments), followed by a short intron and then a constant region exon. The particular kappa constant region used is of the Inv3 alloype, which occurs in 80% of the Caucasian population and 70% of the Black population. The DNA following the termination codon of the C_L segment contains a presumptive poly A signal to allow termination of the mRNA transcript.

Humanized BR55-2 heavy chain. The actual expressed heavy chain gene consists of two adjacent parts: a human genomic gamma 1 constant region built into the vector pVg1, and the humanized heavy chain variable region (V_H), constructed by total gene synthesis in the same manner as described above and cloned into the Xbal site of pVg1. The Xbal fragment, which can be synthesized from four oligonucleotides, encodes the humanized V_H domain, including the J segment and a typical immunoglobulin leader (signal) peptide, which is cleaved off as the heavy chain is secreted. In addition, the segment includes the same 19 base pairs after the J segment that follow the mouse J_H3 segment. The purpose of these nucleotides is to provide a splice donor signal to ensure that the intron between the C_H and the downstream C_H1 is correctly spliced out. The orientation and sequence of the complete segment was verified after cloning into the Xbal site of the pVg1.

Thus, the complete humanized BR55-2 heavy chain gene contains a variable region exon (including leader and J segments), followed by a short intron and then the constant region. The gamma 1 constant region was obtained as a human genomic clone and therefor itself consists of 4 exons - C_H1, H (hinge), C_H2 and C_H3 - separated by 3 introns. The particular gamma 1 constant region used has the Gm allotypic markers, which occur in 60% of the Caucasian population and 100% of the Black population. The DNA following the termination codon of the C_H3 segment contains a presumptive poly A signal to allow termination of the mRNA transcript.

15.5. Transfected cell line

Host cell system. The host cell line was Sp2/0-Ag14 (ATCC CRL 1581). It was isolated as a re-clone of Sp/2/HL-Ag, which was derived from Sp2/HLGK, a hybrid between a BALB/c spleen cell with anti-sheep red blood cell activity and the mouse myeloma line P3X63Ag8. Sp2/0-Ag14 does not survive in HAT medium and has the important characteristic that it does not synthesize or secrete any immunoglobulin chains. For this reason, the cell line is commonly used as a fusion partner in generating hybridomas. It is equally suitable as a host cell line for producing a humanized antibody, because only the transfected immunoglobulin genes will be expressed.

A vial of Sp2/0-Ag14 cells obtained from the ATCC, which will be called here just Sp2/0, was thawed and then passaged several times to produce enough cells to perform DNA transfections. The cells were grown and the transfectants maintained in DMEM medium + 10% fetal calf serum (FCS).

Transfection of cells. Transfection was done by electroporation using a Gene Pulser apparatus (Bio-Rad) at 360 V and 25 µFD capacitance according to the manufacturer's instructions. Before transfection, the light chain- and heavy chain-containing plasmids were linearized using BamHI, extracted with phenol/chloroform, and precipitated with ethanol. All transfections were done using 20 µg plasmid DNA and about 10^7 cells in PBS. The cells from each transfection were plated into one 96-well tissue culture plate. After 48 hours, selective medium was applied.

Cells were selected in DMEM + 10 % FCS + HT media supplement (Sigma) + 1 µg/ml mycophenolic acid. After the wells had become confluent with surviving colonies of cells, medium from each well was assayed for the presence and quantity of secreted antibodies by ELISA. A producing clone from the transfection was grown up to produce antibody for characterization.

To obtain a high-yielding cell line, antibody-producing cells from the transfection were subjected to selection in 50 nM methotrexate. Surviving cells were subjected to increasing concentrations of methotrexate (two fold stepwise) until the level of antibody production reaches the maximum. The best producing cells were then subcloned twice by limited dilution and the highest-yielding clone was selected for production of the respective antibody.

Claims:

1. Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1).

2. Process for the production of anti-idiotypic antibodies according to claim 1 which comprises immunizing mice with BR55-2/murine IgG3-F(ab')$_2$-KLH-conjugate, fusing the murine spleen cells with the murine myeloma cell line SP 2/0, selecting the cultured hybridoma cells which produce IgG with an inhibition capacity of more than 95% (inhibition of binding of BR55-2 murine IgG2a to the SKBR5 cell line), purifying and isolating the anti-idiotypic antibody.

3. Use of anti-idiotypic antibodies according claim 1 for the quantitative determination of monoclonal antibodies, their derivatives or fragments with binding specifity of BR55-2.

4. Use of anti-idiotypic antibodies according claim 1 for the quantitative determination of monoclonal mouse/human chimeras of BR55-2 and of fully humanized variants of BR55-2.

5. Use of anti-idiotypic antibodies according claim 1 for a single step immunopurification of variants of BR55-2.

Abstract:

Monoclonal murine internal image anti-idiotypic antibodies (Ab2) to monoclonal antibodies BR55-2 (Ab1), process for their production and their use for quantitative determination and purification of Mabs with binding specifity of BR55-2.

SR/hz

Figure (6) to accompany abstract

Figure 1
SDS - PAGE

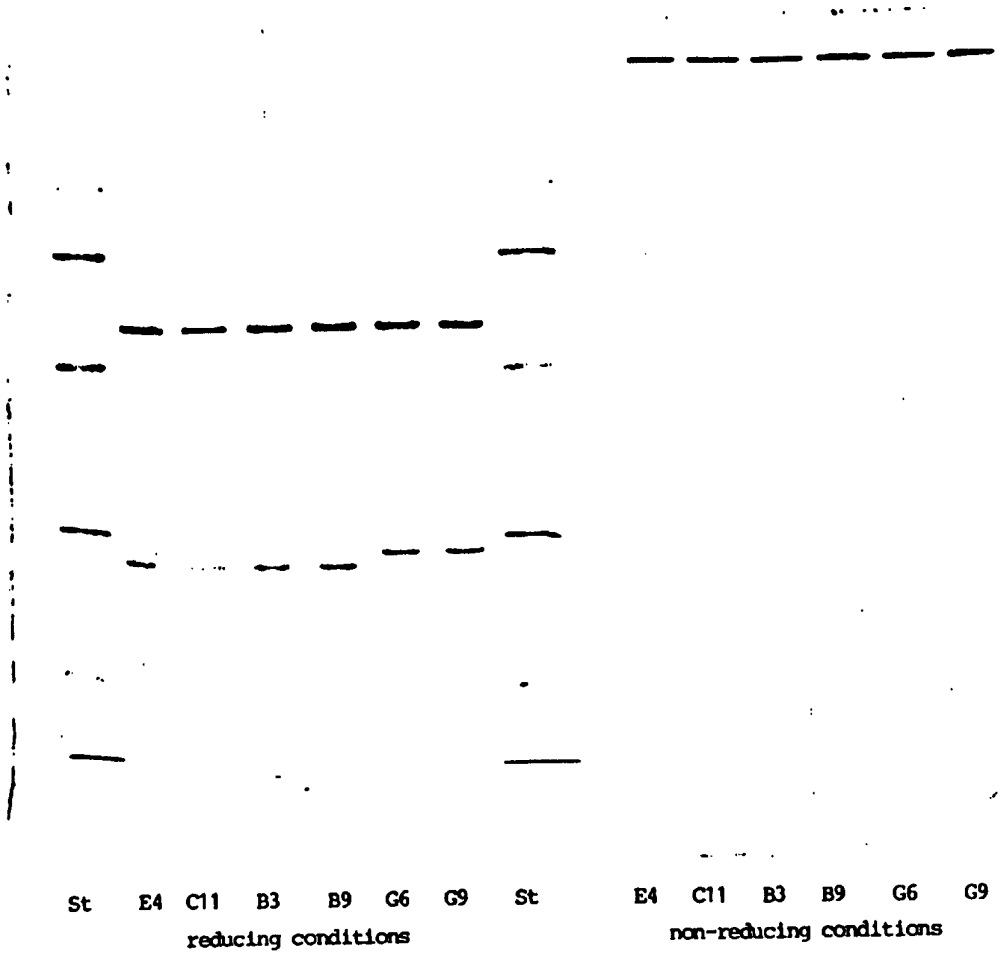
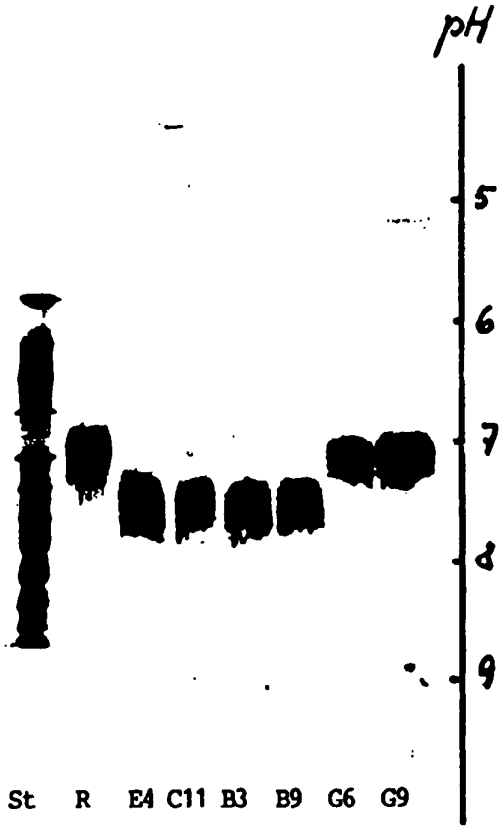
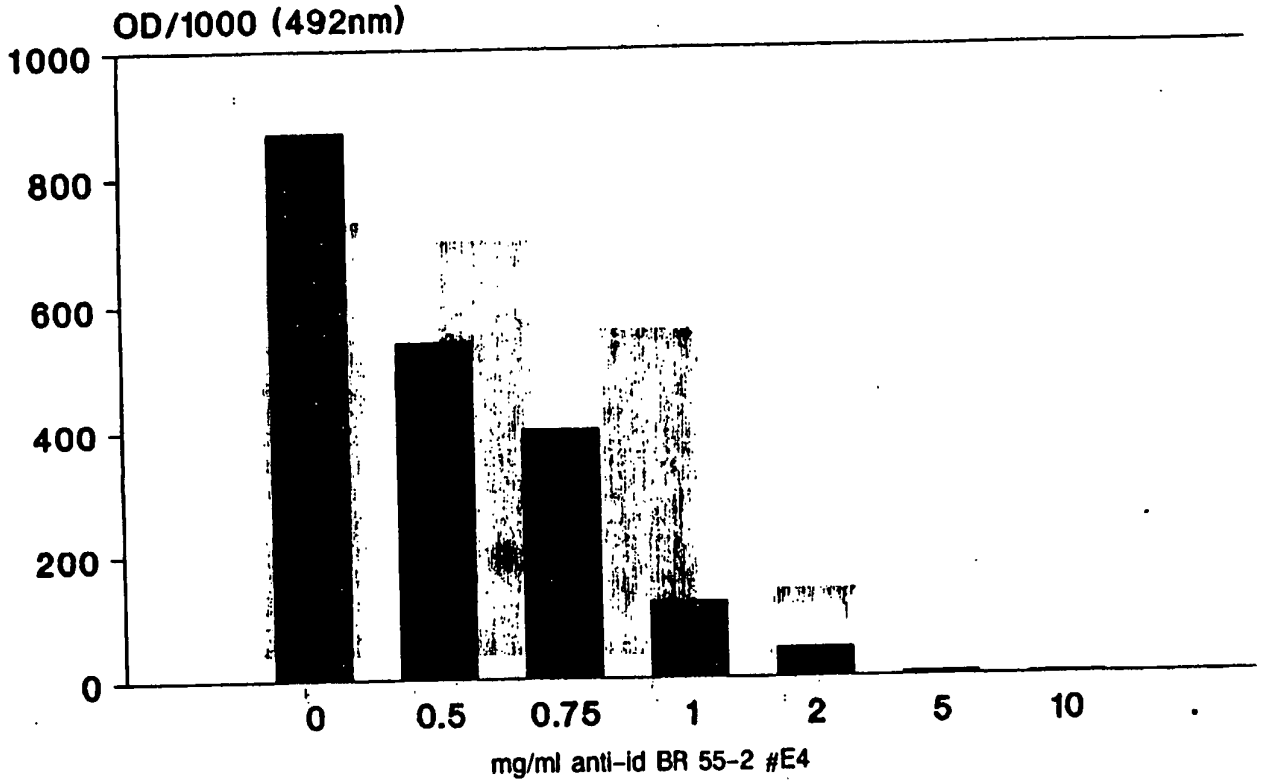


Figure 2
Isoelectric Focussing



St = Standard
R = Reference Mab
E4, C11, B3, B9, G6 and G9 = anti-id BR55-2 Mabs

Figure 3
Binding of BR55-2/murine IgG3 to SKBR5 cell line
Inhibition by anti-id BR55-2 #E4



BR 55-2/murine IgG3: 1 µg/ml

Figure 4
ELISA for the determination of BR55-2/murine IgG3
in human serum - standard curve

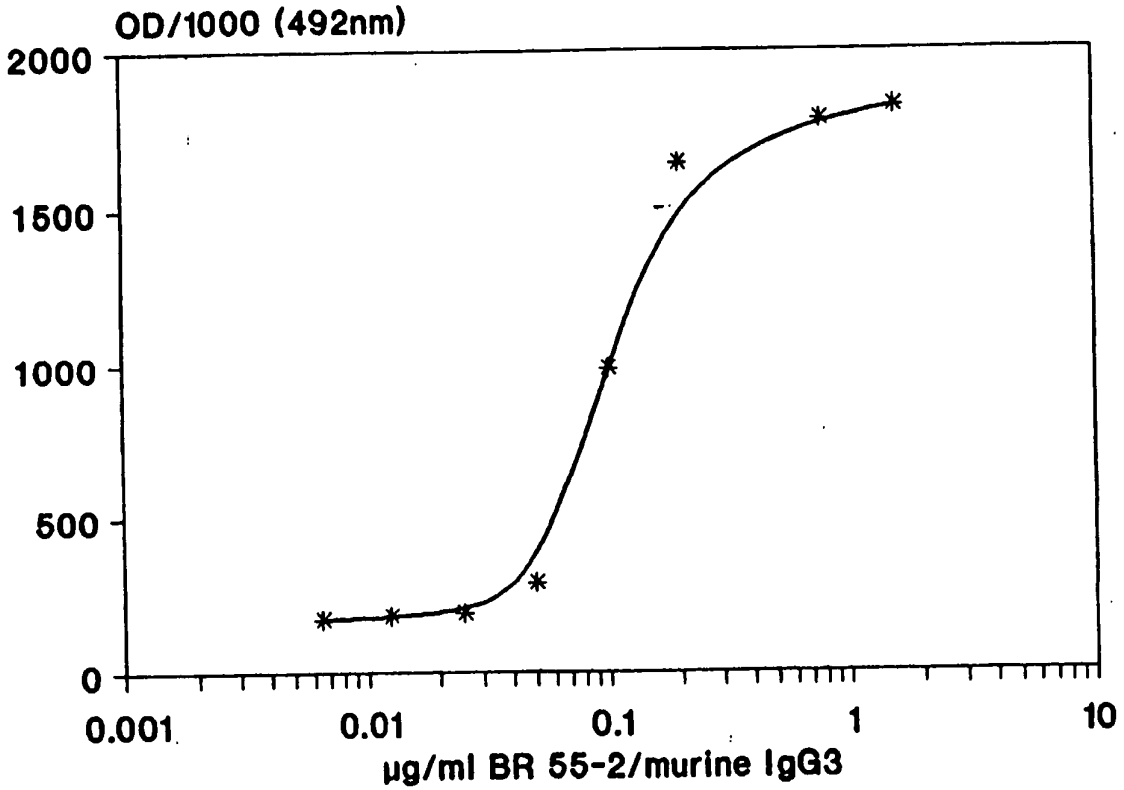


Figure 5
ELISA for the determination of BR55-2/chimeric human IgG1 in human serum - standard curve

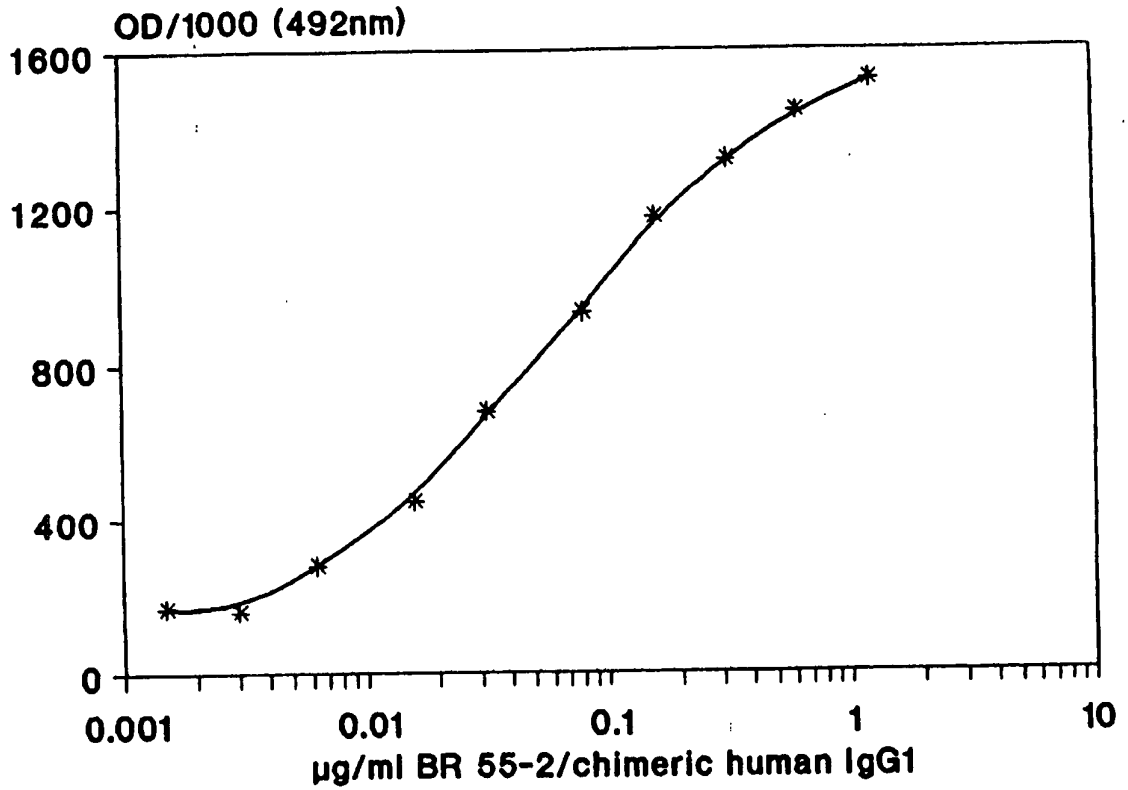


Figure 6
ELISA for the determination of BR55-2/chimeric human IgG3 in human serum - standard curve

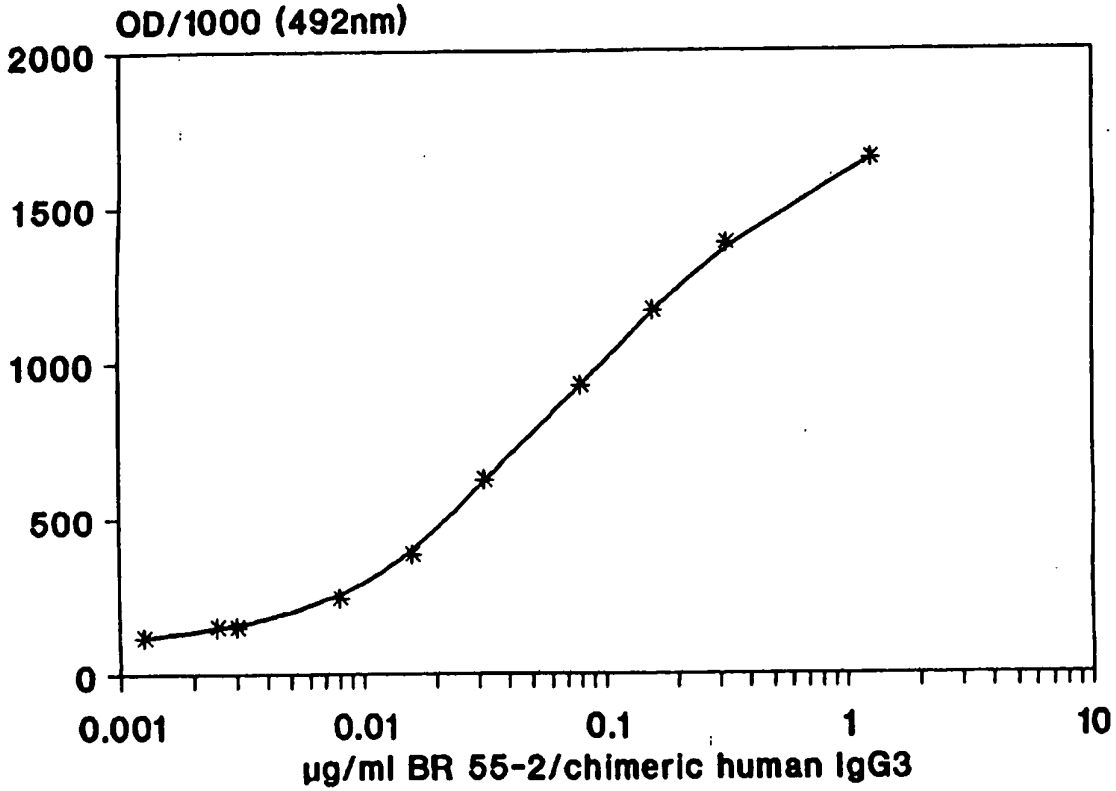


Figure 8
Binding competition of BR55-2/murine IgG3 to a id BR55-2 by BR55-2/chimeric human IgG1 and BR55-2/chim. human IgG3

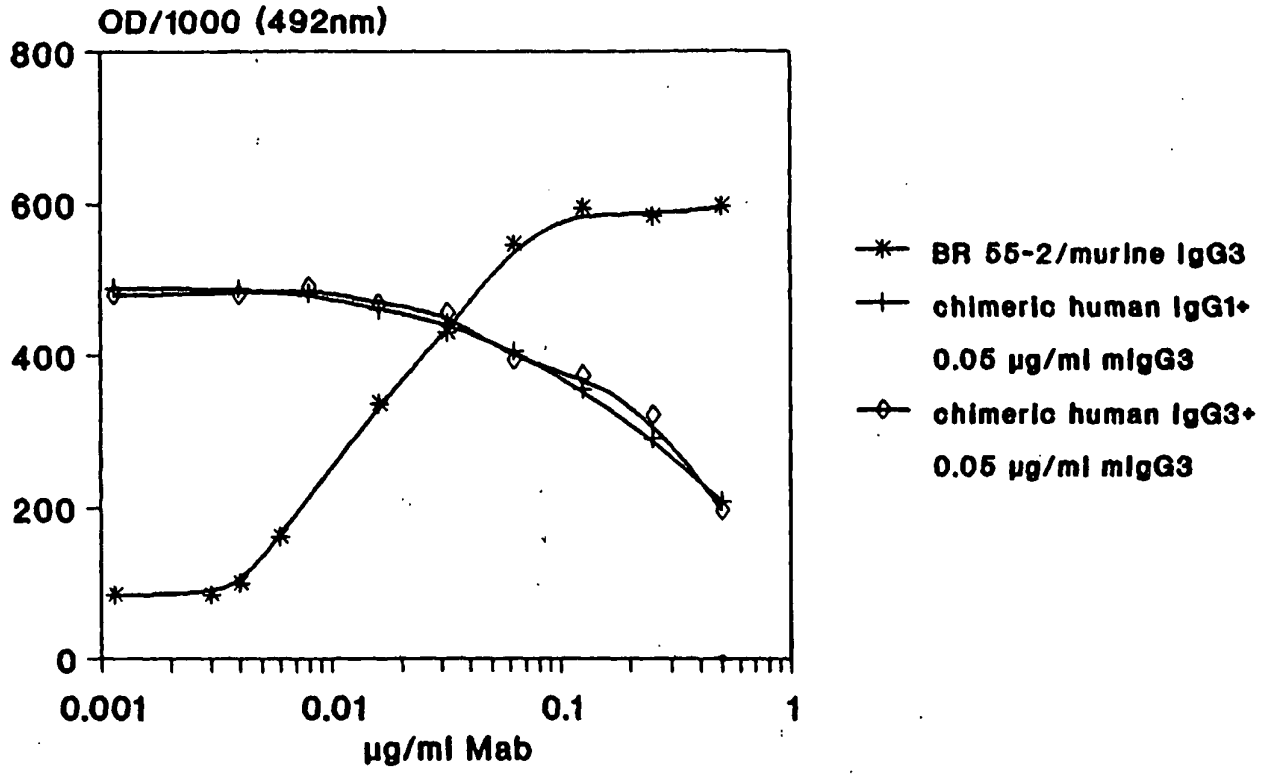


Figure 9
Complement dependent cytotoxicity mediated by BR55-2/murine IgG3
Inhibition by anti-Id BR55-2 #E4

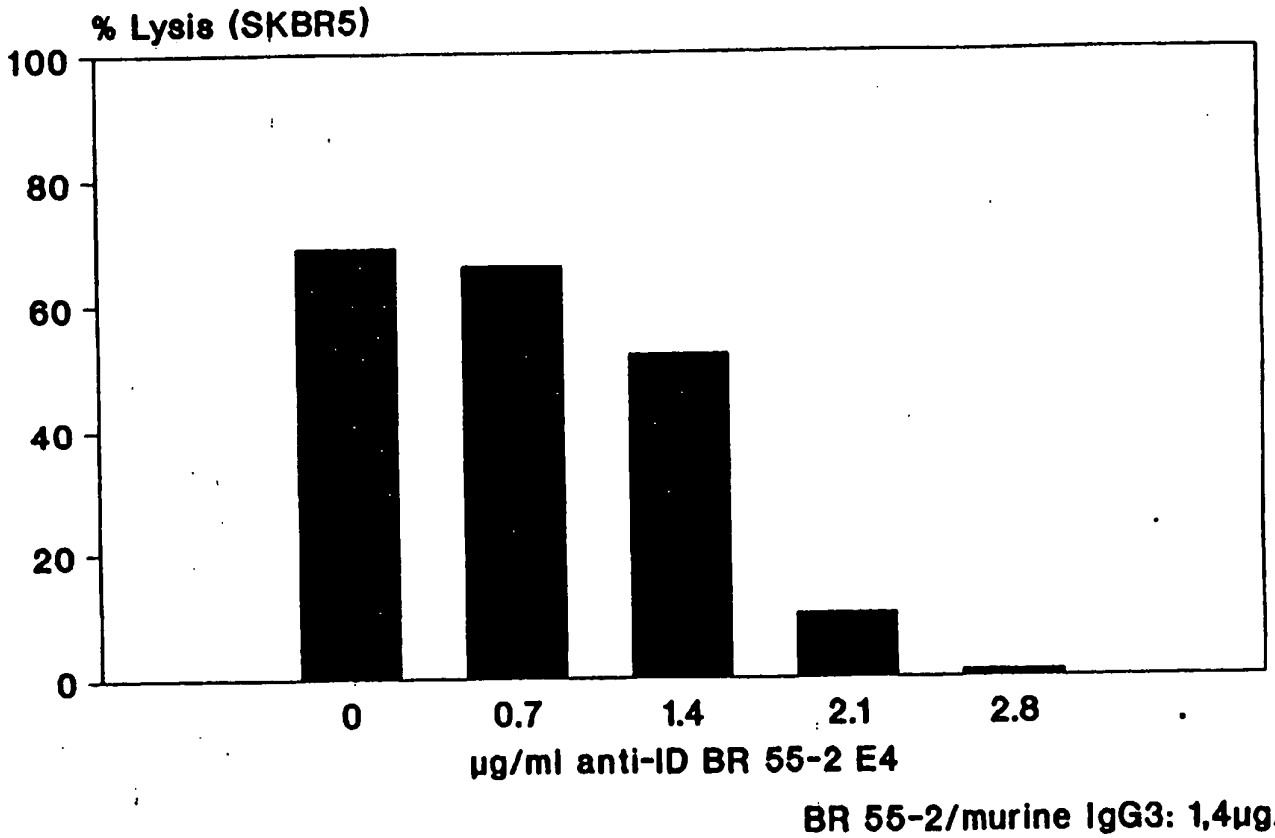
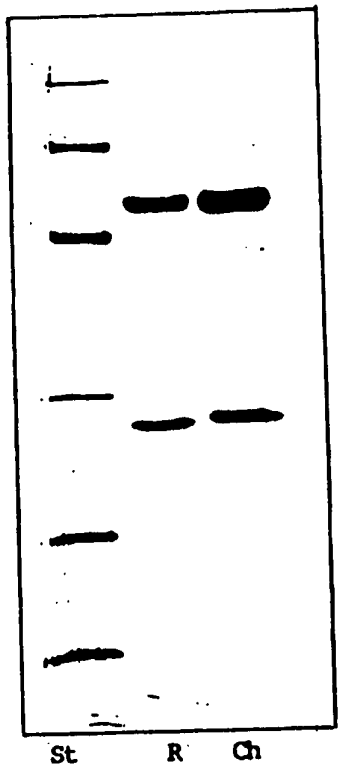


Figure 10
S D S - P A G E
Reducing conditions



St = Standard
R = Reference Mab
Ch = BR55-2/chimeric human IgG1